The relevance of preanalytical factors in metabolomics and lipidomics research

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Omics Technology: Lipidomics and its Pitfalls During the Pre-analytical Stage

Published as: *A. Gil, W. Zhang, J.C. Wolters, H. Permentier, P. Horvatovich, M.R. Heiner-Fokkema, D.J. ReijngoudR. Bischoff. Omics Technology: Lipidomics and Its Pitfalls During the Pre-analytical Stage. Book chapter in: Encyclopedia of Analytical Science, 3rd edition. 2018.*

Abstract

Lipidomics, a derivation of metabolomics, is the most recent member of the omic technologies, and its main objective is the identification and quantitation of the entire collection of chemically distinct lipid species that collectively comprise the lipidome in a cell, tissue, or biological fluid. Despite 20 years of development of the field, common issues regarding the stability of the lipidome during the pre-analytical phase have not been assessed in great detail. With this chapter, our aim is to show the more probable pitfalls of the typical lipidomics strategies and provide the reader with straightforward tools to avoid them.

4.1 Introduction

Systems biology is an evolving multidisciplinary field that attempts to provide a collective insight into complex and dynamic biological processes by understanding and integrating the knowledge of biochemical networks at all levels, from molecules to entire organisms. In this regard, an important milestone during the last decades has been the development of comprehensive methods or so called 'omic' technologies (genomics, transcriptomics, proteomics and metabolomics), that ideally allow to monitor and characterize all members of families of cellular molecules including DNA/RNA, proteins and metabolites in a single analysis.

Metabolomics is the most recent member of the omic technologies and in comparison to genomics and proteomics, which focus on gene expression and protein products, it focuses on the downstream output of biochemical networks within cells. Thus, metabolomics is considered to reveal the last part of the "omics spectrum" and therefore to be a close representation of a cellular or organismal phenotype. Lipids are a subset of the metabolome representing 70% of the entries in the Human Metabolome Database (HMDB) [1]. It is thus no surprise that lipidomics is considered a separate discipline besides metabolomics (Figure 1). Numerous studies have shown that lipids have important signaling functions in addition to their role in cellular membranes and the provision of energy. Lipid-mediated signalling may be altered in widespread human diseases, including cardiovascular disease, diabetes type 2, Alzheimer's disease, inborn errors of metabolism and cancer [1–3]. This has consequently accelerated the development of the lipidomics field mainly focusing on the identification of biomarkers for diagnostic purposes [4,5].

4.2 Lipidomics

The term lipidomics, introduced in 2003 by Spener *et al.* [6] and Han & Gross [7], refers to the identification and quantitation of the entire collection of chemically distinct lipid species that collectively comprise the lipidome in a cell, tissue, or biological fluid.
Usually, lipids produced in an organism are viewed merely as intermediates and/or end points of biosynthesis. Nonetheless, it is becoming clearer that gaining a detailed view of the lipidome (identity, concentration, biosynthesis and function) is important for our understanding of the functioning of cells and organisms within their respective environment. As such, this knowledge forms an integral part of systems biology. Lipids can be studied with two approaches, untargeted lipidomics and targeted lipid analysis [8,9], each with their own strengths and limitations.

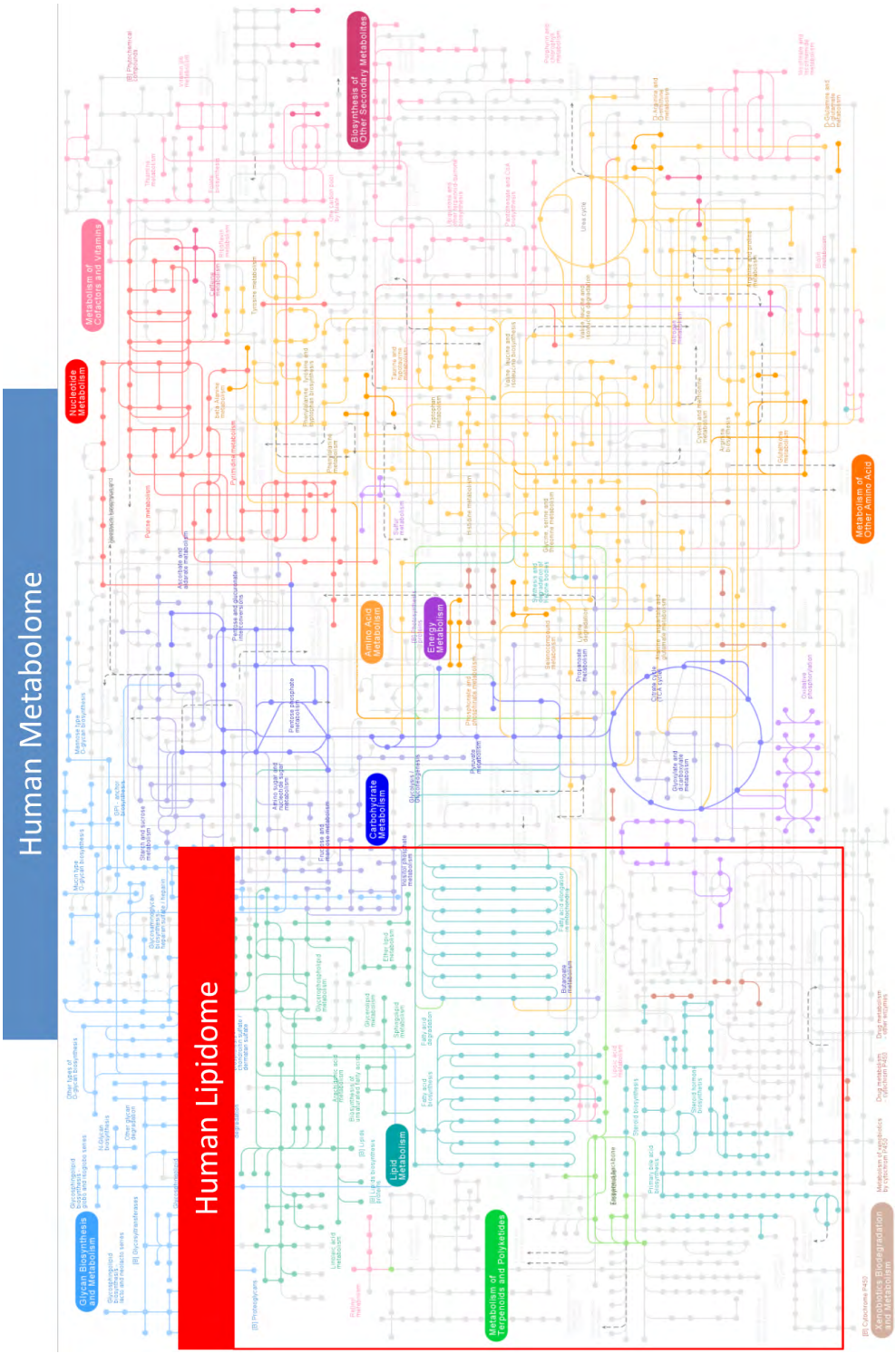

Figure 1. *The human metabolic network. Modified from the KEGG Pathway Database[75].*

4.2.1 Untargeted lipidomics versus targeted lipid analysis

Untargeted or global lipidomics is a hypothesis-free approach that aims at comprehensively measuring levels of all lipid species in a sample, including lipids with structures that have yet to be elucidated. The objective of performing a hypothesis-free analysis is acquiring data with the widest possible coverage of the lipidome followed by identification of those lipids whose relative concentrations change following a perturbation (e.g., a gene knockout, a change in nutrients, amount of specific exercises). Due to its holistic nature (measurement of both known and unidentified lipids), internal standards are not typically used, thus complicating the quantitative determination of absolute lipid levels and making it more useful for the discovery of associations between components of the lipidome.

Targeted lipid analysis, on the other hand, has the goal of quantifying selected lipids that are of specific biological interest, for example as part of a biochemical pathway or network (e.g., prostaglandin synthesis). By using internal standards (usually isotopically labeled lipids), absolute concentrations or amounts can be determined. Targeted lipid analysis requires a thorough understanding of the metabolic pathway or network under study in order to define the lipid species that shall be monitored. In contrast to untargeted lipidomics, sample preparation can be tailored to enrich the lipids of interest while removing highly abundant interfering species. In addition, since all analytes are defined from the very beginning, analytical artifacts are not carried through to the downstream data analysis [10]. Thus, absolute concentrations of lipids in the sample can be determined with high accuracy, precision, sensitivity, and specificity (Figure 2). Since the targeted analysis offers a quantitative picture of selected compounds in a specific part of the lipidome, it is often used as a complementary tool to untargeted lipidomics, notably to follow up on a discovery lipidomics study.

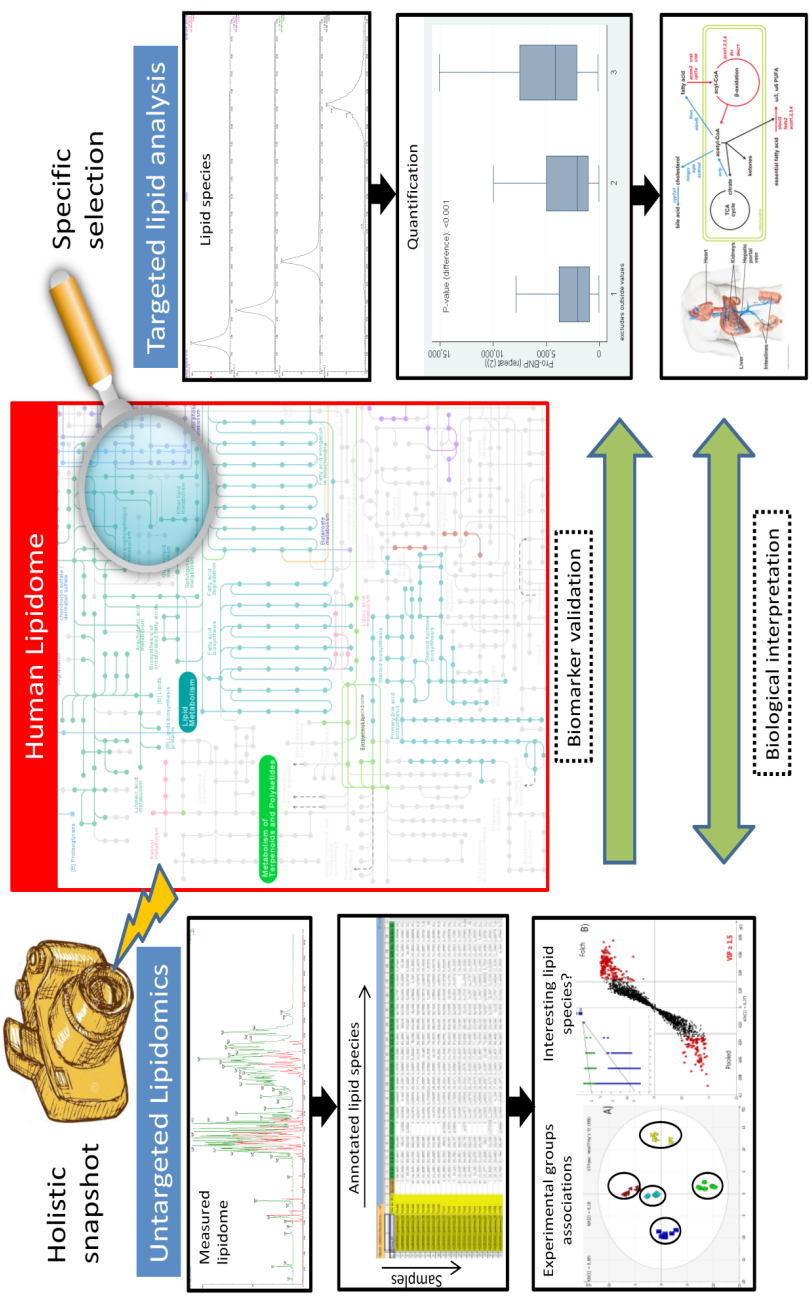

Figure 2. *Differences and similarities between untargeted lipidomics and targeted lipid analysis.*

The general pre-analytical steps for both untargeted lipidomics and targeted lipid analysis are rather similar (Figure 3), while data analysis differs significantly. However, there is a lack of awareness on how lipid stability is affected during sample preparation. Thus, below we focus on common pitfalls in typical lipid analysis (untargeted and targeted) workflows and suggest ways to avoid them.

The stability of lipid molecules, i.e. their susceptibility to chemical degradation during sample handling, is highly related to their structural features. Ester bonds such as those linking fatty acid groups in glycerophospholipids are susceptible to hydrolysis [11]. Saturated fatty acid groups (e.g., myristic acid) are rather stable, while lipids containing unsaturated fatty acids, with one or more double bonds (e.g., dioleic acid), are susceptible to oxidation in the presence of air (oxygen), which is enhanced by exposure to light and the presence of heavy metal ions (notably Fe^{2+}) [11].

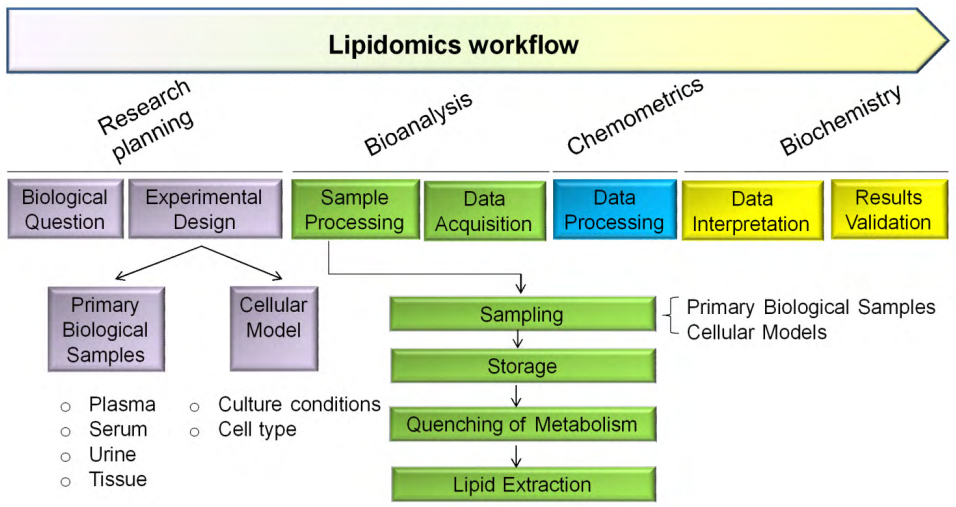

Figure 3. *Elements of a general experimental design and workflow for lipid analysis (untargeted and targeted).*

4.3 Lipid stability

Temperature and pH are critical variables during the pre-analytical phase for any lipid analysis. Temperatures between 4 and 25 °C and pH values ranging from 2 to 8 are typical conditions during lipid analysis. Moreover, chemical environments rich in water and oxygen, the presence of light and sample handling times up to 24 h are common in lipid analysis. Not

surprisingly, all of these conditions might be associated with chemical degradation processes affecting lipid species present in almost any sample matrix, as well as in plain standard solutions. Although there are guidelines for dealing with stability as a key issue in the validation of bioanalytical methods, these usually focus on single analytes [12]. Particularly for untargeted lipidomics studies, sample stability is far more difficult to assess, given the complexity of the samples that are composed of hundreds to tens of thousands of analytes of varying chemical composition and stability. Some studies tried to tackle this problem for proteomics and metabolomics but there is no consensus on how to approach stability issues for complex "Omics" samples [13,14].

The stability of lipidomes under different conditions has not been assessed in great detail despite 20 years of development in the field [15]. The main reason for this is due to limitations in throughput and the lack of suitable reference materials, since it is not possible to follow the typical strategy of targeted lipid analysis in which isotopically labeled internal (homologue) standards are used for each analyte under study. A strategy to overcome this issue might be the use of standardized reference lipidomes for comparison purposes. However, stability assessment is mandatory in lipidomics to produce reliable and reproducible results. In this sense, general considerations must be taken into account in order to minimize the breakdown of lipids species across the workflow, as discussed below.

4.4 The lipidomics workflow and possible pitfalls

Lipidomics is increasingly used in numerous research fields, including food and nutritional [16,17], clinical [18,19], biological [20], pharmaceutical [21] and biotechnological [22] disciplines. Next to high-quality analytical instrumentation and well-trained laboratory staff, it is critical to have experienced personnel in data processing and statistical data analysis as fundamentals to the success of lipidomics projects. Since lipidomics aims to be a comprehensive methodology, all manipulations of the sample from the pre-analytical phase to the analysis of the data will have an effect on the final result. Consequently, standardization of procedures is a prerequisite for obtaining reliable results.

Since lipidomics is a field of research derived from metabolomics, it basically follows the same experimental design and workflow. As shown in Figure 3, the pre-analytical phase of a typical lipidomics/metabolomics research project consists of two major steps. First, the phase of defining the biological/clinical question and the corresponding experimental design, and second, the bioanalytical phase during which sampling, storage, sample handling, metabolism quenching, lipid/metabolite extraction and LC-MS(/MS) protocols need to be

defined and executed. These processes must be strictly controlled and performed according to standardized procedures, to avoid undesired alterations of the samples that may lead to biased results.

4.4.1 Experimental design

4.4.1.1 Lipidomics in body fluids

One of the critical steps in lipidomics, as in any other "Omics" approach, is the selection of appropriate samples to address the biological question. Currently, for clinical lipidomics research, tissue or biofluid samples are used [23]. Due to the ease of collection, plasma, serum and urine have been widely used for the prognosis or diagnosis of many diseases. Additionally, blood (serum and plasma) and urine are integrative biofluids that reflect changes in the (patho)physiology of the entire organism in a single sample [24], which may, however, be problematic, as localized changes due to early disease development may be difficult to detect.

Serum is obtained by allowing the natural process of clotting to occur in whole blood at room temperature (possibly with the addition of clotting activators), followed by centrifugation to isolate the supernatant, while plasma is produced by the removal of cells in a centrifuge after treatment of the blood sample with an anticoagulant to prevent clot formation [24,25]. Although blood is a common biofluid used for biomarker discovery, the degree of natural variation for many lipids remains poorly understood, thus complicating the interpretation of whether observed lipid fluctuations are due to (patho)physiological changes related to a disease, therapeutic interventions or other effects such as nutrition, amount of physical exercise or genetic factors. Few attempts have been made to characterize differences in the lipidome between serum and plasma. Ishikawa *et al.* [26] explored matrix-, age-, gender and stability-associated differences in the lipid profile of plasma versus serum. They found that the clotting process influences the serum lipid profile, since lysophosphatidylcholines (lysoPC) and diacylglycerols (DAG) showed increased levels in comparison to plasma samples. Many sphingomyelin species were significantly higher in females than in males indicating an important gender-associated difference that was independent of age and matrix (plasma and serum). Age-associated differences were more prominent in females than in males in both matrices. Levels of many triacylglycerols were significantly higher in elderly females than in young females. Finally, the authors also demonstrated the negative effect of freeze-thawing of samples on plasma and serum lipidomes, as most lipids showed reduced concentrations after

freeze-thaw cycles. Even when assessing within-subject variance of the plasma lipidome, Begum *et al.* [27] found important changes related to the effect of the circadian rhythm. This emphasizes the need to determine the natural variability of the concentration of lipid species that might be used as baseline values to better identify clinically relevant disease markers.

Urine contains much lower biomolecule concentrations than plasma or serum, notably less protein. Urine contains low-molecular-weight metabolites at rather low concentrations [24] but often with considerable biological variability. Fernández-Peralbo *et al.* [28] proposed that the human urinary metabolome is potentially more complex than serum and plasma if all potential endogenous metabolites and exogenous compounds originating from the intake of nutrition, the gut microbiome and the environment are considered. Lipidomics has been used as a strategy to discover biomarkers for disease diagnosis in urine. Byeon *et al.* [29] explored the lipidome of patients with Gaucher disease (GD) by evaluating the difference between urine and plasma after β-glucocerebrosidase enzyme replacement therapy. They found that in both plasma and urine, all the measured monohexosylceramide (MHC) lipid species tended to be increased in patients when compared to healthy controls. More important was the fact that the concentration of these lipid species decreased after receiving the therapy, implicating that MHC lipid species might be excellent candidates in developing an early diagnostic test for GD in both plasma and urine. Despite this successful example, the metabolic homeostasis of the lipidome in urine, plasma or serum is far from established, as little work has focused on understanding the natural variation of the lipidome in humans over time.

4.4.1.2 Lipidomics in cellular models

In vitro lipidomics studies in cellular models offer some advantages over human or animal studies, including easily controlled experimental variables, greater reproducibility and lower cost. Factors such as age, gender, between-subject variation, and population control baseline values are not relevant when it comes to in vitro studies in cell cultures [30]. Instead, issues with culture conditions, the number of replicates and most importantly the difficulty of translating results to the in vivo situation are among the factors that need to be considered for in vitro studies in cellular model systems. Cells need to be cultured prior to any sample preparation procedure and culture conditions can have an effect on the cellular lipidome. To avoid variation, cells need to grow under standardized and controlled conditions. Seeding density, the culture medium, incubation conditions, harvesting and washing steps are the most important variables that must remain constant. Later, during the collection of cells, extensive washing steps are typically required to avoid remnants of culture medium and

buffers that might affect the lipidome. There is currently a lack of information on how these variables might affect cellular lipidomes. Nonetheless, lipidomics has been used extensively for understanding/interpreting biological responses to diverse stimuli in cell culture systems.

Escherichia coli has been studied extensively due to its importance as a human pathogen. It is among the best-understood organisms and has served as a valuable model for the study of numerous biological processes, including cellular metabolism [31]. *Saccharomyces cerevisiae* (baker's yeast) is another widely studied model in lipidomics research, since it is a simple unicellular eukaryotic organism, containing approximately 6000 genes most of which have orthologues (genes in different species that evolved from a common ancestral gene by speciation) in humans. Taking the high conservation of biological processes and cellular structures throughout eukaryotic systems into account [32], it is possible to gain insight into the human lipidome by using yeast as a model.

Mammalian cells represent another crucial target of lipidomic studies. Human fibroblasts, human breast cancer cell lines, Madin–Darby canine kidney (MDCK) cells, hepatocytes and Chinese hamster ovary (CHO) cells have all been used to gain an overview of the lipidome in eukaryotic cells. Despite the central role of lipids in cell biology, efforts to systematically define changes of the lipidome in mammalian cells in response to normal cellular function or perturbations are rarely found [33]. While there is considerable knowledge about the biochemical pathways that lead to the synthesis and degradation of the major lipid classes, there is comparatively little research on the interactions between these pathways under normal and pathophysiological conditions. Dennis *et al.* [33] characterized changes in the lipidome of macrophages in response to Kdo2-lipid A, a highly specific Toll-Like Receptor ligand that mimics bacterial infection. By quantitatively monitoring the changes, they discovered lipid species not previously reported in macrophages including dihomoprostaglandins, ether-linked triglycerides, ether-linked phosphatidylinositol, ether-linked phosphatidylserine, phosphatidylthreonine, N-acyl phosphatidylserine, and deoxyceramides. This illustrates the potential of lipidomics as a discovery tool.

4.4.2 Bioanalysis

4.4.2.1 Sample processing

As discussed above, a meticulous study design and careful optimization of pre-analytical steps are vital to ensure the quality of the data generated by the subsequent analyses. In

lipidomics, the bioanalysis phase is divided into 4 major steps comprising sample collection (typically blood and/or urine), sample storage, sample handling and lipid extraction (Figure 3).

4.4.2.1.1 Sample collection

Probably the most important part of any large-scale lipidomics study is collection of the sample set. The sampling phase is frequently embedded in the clinical workflow and therefore is one of the less standardized processes. Thus, whereas a failed LC-MS analysis can be repeated or excluded in extreme cases, inconsistencies during sample collection may go unnoticed and invalidate the entire study [24,34]. The impact of devices used for obtaining and storing the samples on the analysis is often overlooked. They can be a source of contaminants, particularly polymers such as polyethylene glycols and phthalates, which may interfere with the subsequent analyses and affect the results [24].

Besides the storage devices, the sampling procedure by itself may affect the result. Preparation of serum requires activation of the blood clotting process, during which a range of enzymes is activated and which may take 30-60 min at room temperature increasing the risk of enzymatic conversions and other degradation processes [25,35]. In order to avoid this as much as possible, it is recommended to clot the blood at reduced temperatures (4 °C), although enzymatic activity is not completely inhibited until temperatures below −56 °C are reached [24].

Plasma preparation from blood, on the other hand, requires the use of anticoagulants such as heparin, sodium citrate or ethylenediaminetetraacetic acid (EDTA) followed by a centrifugation step at 4 °C to separate plasma from the other blood components including red and white blood cells as well as platelets. Platelet activation is associated with significant changes to membrane lipids, and formation of diverse bioactive lipids that play essential roles in hemostasis. Therefore, the use of procedures to prepare platelet-rich and platelet-poor plasma samples must be considered when performing lipid analysis [36]. Heparin is a known platelet activator that may have an important influence on changes in the lipidome of plasma samples [37]. However, anticoagulants may introduce analytical bias and their effect on the lipidome should be evaluated before large-scale sample collection and biobanking [35]. Gonzales-Covarrubias *et al.* [38] found that the effect of citrate, EDTA, and heparin on solution pH or ionic strength may have an important effect on decreasing or increasing the LC-MS response of lipids. They argue, for example, that during the extraction procedure a high ionic strength might increase protein denaturation and consequently increase the release

of lipids from lipoproteins. Furthermore, the molecular weights of citrate and EDTA are in the same range as endogenous low molecular weight fatty acids (FA), so these anticoagulants might affect the analysis, if targeting this type of FA is the purpose of the study. Lipids tend to form adducts with sodium and potassium ions in the absence of chelating agents leading to ionization suppression effects upon electrospray ionization mass spectrometry [39]. Gonzales-Covarrubias *et al.* [38] suggest that some of the observed differences in LC-MS signals in relation to the use anticoagulants might be due to the presence of lithium, as heparin is usually prepared as a lithium salt. This is in agreement with the observation of other researchers that have shown that the presence of lithium-heparin affects the LC–MS response of small molecules differentially [38,40].

Hemolysis during sampling is another possible caveat that may affect the overall lipid profile. Previously, Yin *et al.* [34] showed that approximately 18% of the detected mass signals in a non-targeted metabolomics experiment are affected by hemolysis. Hemolytic samples usually contain increased concentrations of lipids mainly originating from the cellular membranes of erythrocytes [41]. Moreover, the release of intracellular components during the hemolytic process is another major issue that must be avoided, since active enzymes, that might significantly alter the lipidome, may also be released.

For urine analysis, the sampling procedure and sampling time can vary significantly. To evaluate the global status of an individual's lipidome, it is preferable to use 24 h urine sampling to avoid the large variation in lipid profiles with shorter collection periods [28]. Furthermore, treating urine samples with additives to increase lipid solubility and/or to minimize interaction with container surfaces is essential. As an example, Silvester *et al.* [42] established that by adding surfactants, urine metabolites with lipophilic characteristics could be quantified without losses due to unspecific adsorptive processes to the surface of containers, in which the samples were collected, stored or processed. Surfactants may, however, affect the subsequent LC-MS analysis leading to ionization suppression and interfering signals as well as to accumulation on the LC column. The addition of preservatives, such as boric acid or sodium azide, is a common procedure for urine samples to prevent bacterial growth during storage [43]. However, the effect of such reagents on the urine lipidome has not yet been addressed.

For cellular models, sampling involves separating the cellular material from the cell culture medium, and usually a subsequent quenching process to avoid enzymatic degradation of lipid species (discussed below). For mammalian cells, there are some special considerations

depending on whether one uses adherent or non-adherent cells. For non-adherent cells, the pellet can be obtained by centrifugation or filtration. For adherent cells, trypsin, EDTA or scrapers are required to detach the cells from the surface of the culture dish [44]. The influence of this process on lipid profiles has not been addressed. Furthermore, culture flasks are usually made of plastic materials that can introduce contaminants, which may affect lipid analysis.

4.4.2.1.2 Sample storage

Storage is another important step of the pre-analytical phase, as sample content may be altered during storage. Irrespective of the type of sample, be it biofluids, tissues or cells, storage should be done at the lowest temperature available, typically at -80 °C, prior to any further manipulation of the samples [45]. This helps to inhibit enzymatic activity, and to reduce the rate of oxidation and hydrolytic degradation of lipids containing unsaturated fatty acids. It is furthermore recommended to minimize the number of freeze–thaw cycles and to rapidly freeze (ideally using liquid nitrogen) and store pre-aliquoted samples to decrease the potential for degradation as much as possible [25,28]. Particularly for cells, controlled freezing in the presence of cryoprotective solvents must be done in order to maintain the integrity of the cellular membrane, thus avoiding changes in the intracellular lipidome [45].

4.4.2.1.3 Quenching of metabolism

Lipid stability may be affected by chemical processes as well as by enzymatic degradation. Notably remaining enzymatic activity in the sample may alter the lipidome during storage and/or sample preparation, for example during freeze-thaw cycles [25]. Cellular metabolism adjusts rapidly to changes in the environment. Since lipids are coupled through metabolic networks, these changes can quickly generate important alterations in the lipid profiles [46]. Therefore, another important step during the lipidomics workflow, particularly for cell and tissue samples, is quenching of enzymatic activity. Quenching aims to stop metabolism and prevent the turnover of lipids, maintaining their concentrations at the original level. Rapid quenching of metabolism is usually achieved through fast changes in temperature or pH [47].

Quenching procedures were originally developed for and applied to yeast, but have later also been used in studies of different bacteria, mammalian cells, tissues and biofluids [44,48]. Quenching may be achieved by a rapid change of temperature to either low (e.g. below -40 °C) or high temperatures (e.g. above +80 °C), or by switching to an extreme pH, i.e. either alkaline (e.g. KOH or NaOH) or acidic (e.g. perchloric acid, HCl or trichloroacetic acid) [49]. However, the most widely used method is using cold methanol while maintaining the

temperature below -20 °C, followed by collection of the cells with a centrifugation step at 0 °C prior to lipid extraction [44,48]. Although quenching is performed to avoid the enzymatic turnover of the analytes under study, the extreme physical-chemical conditions may lead to complications such as poor compatibility with the subsequent MS methods and more importantly, chemical degradation of some lipid species mainly through hydrolysis of the ester bonds [28,50].

Quenching is a highly sample- and cell-dependent procedure. Differences in cellular composition (especially cell membrane and cell wall structure) and cell size, together with the ratio of methanol to water in the quenching solution may influence the efficiency of the procedure [51]. For lipidomics studies in cells, quenching is a common procedure, whereas for biofluids it is generally omitted, since it is considered to induce changes in the samples [28]. However, in the case of biofluids, sample alteration subsequent to collection is also possible due to residual enzymatic activity. Even in methanolic solutions, some enzymes are prone to remain active, thus, monitoring of substrates for enzymes (e.g., glutamyltransferase, alkaline phosphatase and urease) is recommended since it has provided useful insight into residual enzymatic activity during the pre-analytical phase [25,52].

4.4.2.1.4 Lipid extraction

Post-collection sample handling and preparation are critical during the pre-analytical phase of lipidomics studies, since minor modifications in these processes may result in unwanted and unexpected changes, which can be confused with significant biochemical alterations [53]. For untargeted lipidomics analyses, an ideal sample-preparation method should be (1) unselective, (2) simple and fast with a minimal number of steps and (3) reproducible. In contrast, an ideal sample-preparation method for targeted analysis should be (1) as selective as possible for the lipid(s) under study, (2) reproducible and (3) allow implementation of preconcentration and/or clean-up steps, if required [28].

The necessity of disruption steps prior to lipid extraction makes the handling of solid (tissue) samples more complex in comparison to biofluids. The use of liquid nitrogen to snap-freeze the samples is a common procedure that minimizes stability issues and makes physical manipulation easier. The next step is homogenization of the sample, required to ensure efficient and reproducible extraction of the lipidome. Frozen solid samples can be homogenized with a mortar and pestle by manual grinding, or by using bead beater homogenizers together with an appropriate extraction solvent [54]. Sample handling of biofluids is mainly focused on increasing the efficiency of lipid extraction by using different solvent systems, making it more straightforward than solid samples. As mentioned before, plasma/serum and urine are

the most typical samples for lipidomics studies. However, specialized fluids, such as tear fluid, cerebrospinal fluid and synovial fluid have recently been investigated as well (Table 1).

Regardless of the type of sample, each matrix represents a different challenge for the analyst since its chemical composition (proteins, polar metabolites and lipid levels) determines the best way to proceed with the sample preparation. For instance, plasma and serum are lipid-rich biofluids, containing more than 17,000 species of lipids and fatty acids whose concentrations range between 4 pM and 2 mM [54–56]. The high concentration of proteins is known to be the major challenge for lipidomics in plasma and serum, as proteins are considered the main class of interfering compounds in these types of samples. Although the metabolome of human urine is rather complex, the lipidome is limited in complexity containing low concentrations (ranging from 0.1 to 1450 nM) of approximately 800 species of lipids and fatty acids [57,58]. Additionally, degradation products of metabolites from food and/or drug intake represent a source of interference in urine samples. Therefore, whereas in plasma and serum, protein removal is important during sample preparation and is usually achieved by precipitation with the organic solvents used for lipid extraction, for urine, concentration processes such as lyophilization and/or solid-phase extraction must be considered due to the generally low concentration levels of lipids.

The extraction of all lipid species in a comprehensive manner remains an active area of research in the lipidomics field. Liquid-liquid extraction is the standard technique for lipid analysis. The most commonly used methods for lipid extraction were introduced by Folch *et al.* [59] and by Bligh & Dyer [60]. Over time, these methods have been modified according to particular needs [61,62], while maintaining the basic principle of using different proportions of chloroform/methanol that separate into an upper methanol-rich layer, containing hydrophilic compounds, and a lower chloroform-rich layer mainly containing lipids. Despite the popularity of these two methods, a major drawback is the need of retrieving the lipids from the lower chloroform-rich layer, which may lead to contamination that can compromise the analysis due to the presence of insoluble material accumulating at the interphase [2]. To overcome this problem, two alternative biphasic solvent systems were introduced more recently. Matyash *et al.* [63] described the methyl tert-butyl ether (MTBE) extraction method and more recently Löfgren *et al.* [64] proposed the butanol-methanol (BUME) method. These methods have the advantage that the lipid-rich organic phase forms the upper layer of the biphasic system. The extraction methods have been successfully applied for the extraction of key lipid classes, ranging from phospholipids and glycolipids to fatty acids, di- (DAGs) and tri-acylglycerols (TAGs). However, contradictory reports particularly on the efficiency for extracting polar lipid species, such as glycolipids and lysophospholipids, have appeared [4].

Since current lipidomics focuses on the use of high-throughput methods that allow the analysis of hundreds of samples in the shortest possible period of time (e.g. 96 samples each 3 hours according to Christinat *et al.*) [65], single-phase lipid extractions that improve the recovery of polar lipid species while avoiding the inherent problems of the liquid-liquid extraction methods have recently gained in popularity [4] (Table 1). By denaturing proteins that are later removed by centrifugation, single-phase extractions focus on an 'all-in-one-tube' approach eliminating the need for phase separation. Methanol, butanol, isopropanol and MTBE alone and miscible mixtures thereof have been used as solvents [2,4,64]. However, so far most studies using single-phase extraction methods have focused on the targeted evaluation and recovery of a small set of lipid standards, hence, hindering the assessment of their suitability with respect to coverage of the lipidome in comparison to liquid-liquid extractions [2,4].

Another important issue that has been rarely addressed regarding sample preparation procedures for lipidomics is the lack of standardized criteria to evaluate the suitability of different extraction methods. For targeted lipid analysis, this is typically achieved by validating the analytical method (from the sample processing to the data acquisition step), with respect to selectivity, accuracy, precision, recovery and linearity of the response for a small number of lipid species [4,12]. Given the complexity of the lipidome, it is not possible to apply the same approach to evaluate the suitability of extraction methods for untargeted lipidomics analysis. However, some efforts have been made towards the standardization of a defined set of criteria. Previously, Sarafian *et al.* [66] compared different single-phase and liquid-liquid extraction methods with an untargeted approach. They concluded that simplicity of the extraction procedure, protein removal efficiency, repeatability, lipid coverage and lipid recovery were the most important requirements that an extraction method must fulfill in view of untargeted lipidomics. Based on these criteria, they found that using single-phase extractions mainly based on isopropanol (IPA) as solvent gave repeatable results of the lipidome from plasma samples with enhanced lipid coverage and good recovery. As precipitation of proteins with IPA is widely used in drug analysis, it may be argued that many rather polar compounds will also remain in the supernatant potentially complicating the analysis and affecting the final results. It is fair to say that, despite recent improvements, there is no extraction approach that provides a complete picture of the lipidome due to the structural diversity of its components, which makes it unlikely that a "one size fits all" approach will be developed any time soon, if at all.

Solid phase extraction (SPE) is based on the use of a solid (stationary) phase and a liquid (mobile) phase to improve the selectivity of the analytical method, by capturing specific

classes of lipids with similar physical-chemical properties [54]. Consequently, this is a widespread methodology in targeted lipid analysis. SPE is a well-known sample-preparation method that typically requires a protein precipitation step and is combined with liquid-liquid extraction as additional clean-up step to enrich specific classes of lipids from the biological matrix [54]. Normal-phase silica columns, reversed-phase columns (C8 and C18), and ion-exchange columns (e.g. aminopropyl) are commonly used for the separation and sub-fractionation across a wide polarity range, from low polarity triacylglycerides (TAG) and cholesterol esters (CE) to more polar low molecular weight fatty acids and glycolipids [67,68].

A number of alternative techniques that aid in the extraction process are being used more frequently. Supercritical fluid extraction (SFE), microwave-assisted extraction (MAE) and ultrasound-assisted extraction (UAE) are techniques that decrease extraction times and require lower amounts of solvents [54]. A summary of the most common types of samples and extraction strategies used for lipidomics analysis is shown in Table 1.

Once lipid extracts are prepared, stability remains an issue that needs to be considered. At this point, in order to avoid degradation processes, dividing the extract into aliquots is recommended, as this will reduce the number of freeze-thaw cycles to which samples are subjected. Moreover, the removal of air from the headspace of sample vials containing the lipid extracts, by passing an inert gas, such as nitrogen, over the mouths of the vials prior to sealing them, can help to minimize lipid oxidation.

4.4.2.2 Common analytical platforms in lipidomics

Data acquisition is the next part of the lipidomics workflow once lipid extracts have been prepared (Figure 3). Below, we give a brief overview of the main analytical approaches that are currently used. More detailed accounts can be found in the literature [1,54,69].

Robust analytical techniques that allow the simultaneous measurement of large numbers of lipids in a single biological sample form the basis of lipidomics. Nuclear magnetic resonance spectroscopy (^{1}H and ^{13}C-NMR) and mass spectrometry (MS) are the most commonly applied techniques [70].

NMR spectroscopy has been widely used since the early days of lipidomics research, notably for elucidating lipid structures [69]. The major advantages of NMR-based lipid profiling are the high throughput combined with reproducibility and ease of analysis, besides high information content of the spectra due to simultaneous detection of all molecules in one

snapshot [71]. In contrast, the major drawback of this technique is its limited sensitivity.

Mass spectrometry (MS), combined with effective sample preparation and separation techniques, is undoubtedly the main approach in lipidomics due high sensitivity, specificity, as well as a wide dynamic range. Thus, hyphenated MS techniques have rapidly emerged as the dominant approach for lipid profiling [72]. In conjunction with liquid chromatography (LC), ionization may be achieved using matrix assisted laser desorption/ionization (MALDI), atmospheric pressure chemical ionization (APCI) and electrospray ionization (ESI), the latter being the most popular, since it is a soft ionization technique that allows the generation of molecular ions without excessive fragmentation [54]. While low-resolution mass analyzers like quadrupole ion traps or quadrupoles (IT, Q) are well-suited for targeted lipid analyses, they are suboptimal for lipidomics, due to the high complexity of lipid samples and the need for high mass resolution and mass accuracy. This is why time-of-flight (TOF) and orbital trap (Orbitrap) mass analyzers alone or in combination with IT or Q mass analyzers in hybrid or tandem configurations have become the instruments of choice for lipidomics, as they allow precursor ion selection and fragmentation to acquire MS/MS data of higher specificity [73].

Table 1. *Advantages and disadvantages of extraction methods commonly used for lipidomics studies (according to Teo, et al. [55]).*

	Extraction method	Type of sample	Advantages	Disadvantages	Reference
1.	Single phase extraction	•Serum (Biofluid) •Skin (Solid)	• Easy to perform • Short extraction time • Low cost • No increase in temperature for thermolabile components • No external energy supplied	• Usage of toxic organic solvent • Can be hard to remove organic solvent for analysis	70,71 72
2.	Liquid-Liquid extraction	•Tears (Biofluid) •Serum (Biofluid) •Plasma (Biofluid) •Urine (Biofluid) •Synovial fluid (Biofluid) •Atherosclerotic plaque (Solid) •Skin (Solid) •Tissue (Solid)	• Well-established method • Easy to perform • Cheap apparatus • Short extraction time • Cheap solvents used (e.g. chloroform, methanol, water) • No increased temperature for thermally labile compounds • No external energy supplied	• Large volume of toxic organic solvents • Usually needs more than one type of solvent • Need to remove solvents to avoid interferences with the analysis	73 74–79 65,80–88 89,90 91 92 93–95 96,97
3.	Solid phase extraction	•Serum (Biofluid) •Sputum (Biofluid) •Eye (Solid) •Skin (Solid)	• Easy to perform • Cleans up interfering matrix components • Choice of different SPE modes • No increase in temperature for thermally labile compounds • No external energy supplied • Short extraction time	• Costly SPE cartridges • Need to wash out organic solvents to avoid interferences with the analysis • Can be hard to remove organic solvent for analysis	70,71,78 98 94 72,95
4.	Supercritical fluid extraction	•Plasma (Biofluid)	• No organic solvent • Short extraction time • High extraction efficiency for non-polar components • CO_2 is recyclable • Temperature and pressure controllable • Modifiers can be added to improve polarity for extraction efficiency	• Challenging to operate • Costly • Requires specific equipment	99
5.	Microwave-assisted extraction	•Plasma (Biofluid) •Skin (Solid)	• Easy to perform • Short extraction time • High extraction efficiency • Low consumption of extraction solvent • Temperature and pressure controllable	• Possible loss of volatile organic compounds • Requires specific equipment • Needs an external source of energy	100 101
6.	Ultrasound-assisted extraction	•Blood (Biofluid)	• Easy to perform • Short extraction time • Low extraction solvent volume used • Temperature controllable	• Harmful solvent may be inhaled • Needs an external source of energy • Requires specific equipment • Increased temperature due to frictions can potentially alter compounds	5,102

Despite the separation and identification capabilities of LC-high-resolution-MS for lipidomics analyses, there are important challenges that have to be addressed to get closer to a comprehensive view of a lipidome. Especially, the selection of the chromatographic conditions has an important effect on the type and number of lipids that can be detected. Most LC-MS-based lipidomics studies have been performed by reversed-phase high-performance liquid chromatography (RP-HPLC). Separation is governed by lipophilicity, which is determined by the length of the acyl chains. Thus, lipid species containing longer acyl chains elute later from the LC column than lipids with shorter acyl chains, and lipids with saturated acyl chains elute later than their mono- or polyunsaturated analogs.

Hydrophilic interaction chromatography (HILIC) was more recently introduced for lipid analysis. This technique allows a separation of lipids primarily based on the hydrophilic nature of the head group. Utilization of HILIC in general has grown in popularity thanks to the excellent compatibility with mass spectrometry (enhanced MS sensitivity due to the high organic content in the mobile phase and the high efficiency of spray formation and desolvation) [74]. Since selectivity of lipidome analyses by HILIC and RP is complementary, two-dimensional LC (LC x LC) methods have been established using a combination of both separation modes, thus increasing the coverage of the lipidome from the more polar lysophosphatidylcholines (lysoPC), lysophosphatidylethanolamines (lysoPE), phosphatidylcholines (PC), phosphatidylethanolamines (PE) and phosphatidylinositol (PI) species to the most apolar cholesterol esters (CE) and triglycerides (TG) [54].

4.5 Conclusion

With this chapter, focused on the pre-analytical aspects affecting lipidomics, we provide the reader with background information concerning lipidomics studies, and insights into some of the major limitations. Separation and detection techniques for lipidomics analysis are continuously evolving and being reviewed, but technical aspects related to the pre-analytical stage and stability issues of lipid species have rarely been addressed. Currently, lipidomics is moving towards its use as a diagnostic tool in a regulated clinical environment. Therefore, addressing lipid stability issues and avoiding the introduction of bias during the pre-analytical phase of the analysis is critical for the further development of the field.

References

[1] Sandra, K., t'kindt, R., Jorge, L., Sandra, P., in:, Metabolomics Pract., Wiley-VCH Verlag GmbH & Co. KGaA, Weinheim, Germany, 2013, pp. 137–176.

[2] Löfgren, L., Forsberg, G.-B., Ståhlman, M., Tolley, B., Tigyi, G. J., Sci. Rep. 2016, 6, 27688.

[3] Garcia-Cazorla, À., Mochel, F., Lamari, F., Saudubray, J.-M., J. Inherit. Metab. Dis. 2015, 38, 19–40.

[4] Pellegrino, R. M., Di Veroli, A., Valeri, A., Goracci, L., Cruciani, G., Anal. Bioanal. Chem. 2014, 406, 7937–7948.

[5] Pizarro, C., Arenzana-Rámila, I., Pérez-Del-Notario, N., Pérez-Matute, P., González-Sáiz, J. M., Anal. Chem. 2013, 85, 12085–12092.

[6] Spener, F., Lagarde, M., Géloên, A., Record, M., Eur. J. Lipid Sci. Technol. 2003, 105, 481–482.

[7] Han, X., Gross, R. W., J. Lipid Res. 2003, 44, 1071–9.

[8] McGhie, T. K., Rowan, D. D., Mol. Nutr. Food Res. 2012, 56, 147–158.

[9] Du, F., Virtue, A., Wang, H., Yang, X.-F., Biomark. Res. 2013, 1, 1–17.

[10] Roberts, L. D., Souza, A. L., Gerszten, R. E., Clish, C. B., in:, F.M. Ausubel, R. Brent, R.E. Kingston, D.D. Moore, J.G. Seidman, J.A. Smith, K. Struhl (Eds.), Curr. Protoc. Mol. Biol., John Wiley & Sons Inc., 2012, p. 30.2.1-24.

[11] Saldana, M. D. A., Martinez-Monteagudo, S. I., in:, Appl. Calorim. a Wide Context - Differ. Scanning Calorimetry, Isothermal Titration Calorim. Microcalorim., InTech, 2013.

[12] U.S. Department of Health and Human Services, Food and Drug Administration (HSS-FDA). Guidance for Industry - Bioanalytical Method Validation, Rockville, MD, USA, 2013.

[13] Rosenling, T., Stoop, M. P., Smolinska, A., Muilwijk, B., Coulier, L., Shi, S., Dane, A., Christin, C., Suits, F., Horvatovich, P. L., Wijmenga, S. S., Buydens, L. M. C., Vreeken, R., Hankemeier, T., Van Gool, A. J., Luider, T. M., Bischoff, R., Clin. Chem. 2011, 57, 1703–1711.

[14] Rosenling, T., Slim, C. L., Christin, C., Coulier, L., Shi, S., Stoop, M. P., Bosman, J., Suits, F., Horvatovich, P. L., Stockhofe-Zurwieden, N., Vreeken, R., Hankemeier, T., Van Gool, A. J., Luider, T. M., Bischoff, R., J. Proteome Res. 2009, 8, 5511–5522.

[15] Heiskanen, L. A., Suoniemi, M., Ta, H. X., Tarasov, K., Ekroos, K., Anal. Chem. 2013, 85, 8757–8763.

[16] Smilowitz, J. T., Zivkovic, A. M., Wan, Y. J. Y., Watkins, S. M., Nording, M. L., Hammock, B. D., German, J. B., Mol. Nutr. Food Res. 2013, 57, 1319–1335.

[17] Hyötyläinen, T., Bondia-Pons, I., Orešič, M., Mol. Nutr. Food Res. 2013, 57, 1306–1318.

[18] Zhao, Y.-Y., Cheng, X., Lin, R.-C., Lipidomics Applications for Discovering Biomarkers of Diseases in Clinical Chemistry, 2014.

[19] Hyötyläinen, T., Orešič, M., Bioanalysis 2016, 8, 351–364.

[20] Cajka, T., Fiehn, O., TrAC - Trends Anal. Chem. 2014, 61, 192–206.

[21] Satomi, Y., in:, Lipidomics, Wiley-VCH Verlag GmbH & Co. KGaA, Weinheim, Germany, 2012, pp. 319–328.

[22] Schörken, U., Kempers, P., Eur. J. Lipid Sci. Technol. 2009, 111, 627–645.

[23] Saude, E. J., Sykes, B. D., Metabolomics 2007, 3, 19–27.

[24] Dunn, W. B., Broadhurst, D., Begley, P.,

Zelena, E., Francis-Mcintyre, S., Anderson, N., Brown, M., Knowles, J. D., Halsall, A., Haselden, J. N., Nicholls, A. W., Wilson, I. D., Kell, D. B., Goodacre, R., Nat. Protoc. 2011, 6, 1060–1083.

[25] Vuckovic, D., Anal. Bioanal. Chem. 2012, 403, 1523–1548.

[26] Ishikawa, M., Maekawa, K., Saito, K., Senoo, Y., Urata, M., Murayama, M., Tajima, Y., Kumagai, Y., Saito, Y., PLoS One 2014, 9,.

[27] Begum, H., Li, B., Shui, G., Cazenave-Gassiot, A., Soong, R., Ong, R. T.-H., Little, P., Teo, Y.-Y., Wenk, M. R., Sci. Rep. 2016, 6, 19139.

[28] Fernández-Peralbo, M. A., Luque de Castro, M. D., TrAC - Trends Anal. Chem. 2012, 41, 75–85.

[29] Byeon, S. K., Lee, J. Y., Lee, J.-S., Moon, M. H., J. Chromatogr. A 2015, 1381, 132–139.

[30] León, Z., García-Cañaveras, J. C., Donato, M. T., Lahoz, A., Electrophoresis 2013, 34, 2762–2775.

[31] Rabinowitz, J. D., Expert Rev. Proteomics 2007, 4, 187–198.

[32] Dunn, W. B., Winder, C. L., Methods Enzymol. 2011, 500, 277–297.

[33] Dennis, E. A., Deems, R. A., Harkewicz, R., Quehenberger, O., Brown, H. A., Milne, S. B., Myers, D. S., Glass, C. K., Hardiman, G., Reichart, D., Merrill, A. H., Sullards, M. C., Wang, E., Murphy, R. C., Raetz, C. R. H., Garrett, T. A., Guan, Z., Ryan, A. C., Russell, D. W., McDonald, J. G., Thompson, B. M., Shaw, W. A., Sud, M., Zhao, Y., Gupta, S., Maurya, M. R., Fahy, E., Subramaniama, S., J. Biol. Chem. 2010, 285, 39976–39985.

[34] Yin, P., Peter, A., Franken, H., Zhao, X., Neukamm, S. S., Rosenbaum, L., Lucio, M., Zell, A., Häring, H.-U., Xu, G., Lehmann, R., Clin. Chem. 2013, 59, 833–845.

[35] Denery, J. R., Nunes, A. A. K., Dickerson, T. J., Anal. Chem. 2011, 83, 1040–1047.

[36] O'Donnell, V. B., Murphy, R. C., Watson, S. P., Circ. Res. 2014, 114, 1185–1203.

[37] Cella, G., Girolami, a, Sasahara, a a, Circulation 1999, 99, 3323.

[38] Gonzalez-Covarrubias, V., Dane, A., Hankemeier, T., Vreeken, R. J., Metabolomics 2013, 9, 337–348.

[39] Wedge, D. C., Allwood, J. W., Dunn, W., Vaughan, A. A., Simpson, K., Brown, M., Priest, L., Blackhall, F. H., Whetton, A. D., Dive, C., Goodacre, R., Anal. Chem. 2011, 83, 6689–6697.

[40] Mei, H., Hsieh, Y., Nardo, C., Xu, X., Wang, S., Ng, K., Korfmacher, W. A., Rapid Commun. Mass Spectrom. 2003, 17, 97–103.

[41] Dimeski, G., Mollee, P., Carter, A., Clin. Chem. 2005, 51, 2425.

[42] Silvester, S., Zang, F., J. Chromatogr. B 2012, 893–894, 134–143.

[43] Emwas, A.-H., Roy, R., McKay, R. T., Ryan, D., Brennan, L., Tenori, L., Luchinat, C., Gao, X., Zeri, A. C., J. Proteome Res. 2016, 15, 360–373.

[44] Dettmer, K., Nürnberger, N., Kaspar, H., Gruber, M. A., Almstetter, M. F., Oefner, P. J., Anal. Bioanal. Chem. 2011, 399, 1127–1139.

[45] Duarte, I. F., Marques, J., Ladeirinha, A. F., Rocha, C., Lamego, I., Calheiros, R., Silva, T. M., Marques, M. P. M., Melo, J. B., Carreira, I. M., Gil, A. M., Anal. Chem. 2009, 81, 5023–5032.

[46] Villas-Bôas, S. G., Bruheim, P., Anal. Biochem. 2007, 370, 87–97.

[47] Villas-Bôas, S. G., Højer-Pedersen, J., Akesson, M., Smedsgaard, J., Nielsen, J., Yeast 2005, 22, 1155–1169.

[48] Bolten, C. J., Kiefer, P., Letisse, F., Portais,

J.-C., Wittmann, C., Anal. Chem. 2007, 79, 3843–3849.

[49] Mashego, M. R., Rumbold, K., De Mey, M., Vandamme, E., Soetaert, W., Heijnen, J. J., Biotechnol. Lett. 2007, 29, 1–16.

[50] Aveldaño, M. I., Horrocks, L. A., J. Lipid Res. 1983, 24, 1101–1105.

[51] Sellick, C. A., Knight, D., Croxford, A. S., Maqsood, A. R., Stephens, G. M., Goodacre, R., Dickson, A. J., Metabolomics 2010, 6, 427–438.

[52] Bernini, P., Bertini, I., Luchinat, C., Nincheri, P., Staderini, S., Turano, P., J. Biomol. NMR 2011, 49, 231–243.

[53] Theodoridis, G. A., Gika, H. G., Want, E. J., Wilson, I. D., Anal. Chim. Acta 2012, 711, 7–16.

[54] Teo, C. C., Chong, W. P. K., Tan, E., Basri, N. B., Low, Z. J., Ho, Y. S., TrAC - Trends Anal. Chem. 2015, 66, 1–18.

[55] Psychogios, N., Hau, D. D., Peng, J., Guo, A. C., Mandal, R., Bouatra, S., Sinelnikov, I., Krishnamurthy, R., Eisner, R., Gautam, B., Young, N., Xia, J., Knox, C., Dong, E., Huang, P., Hollander, Z., Pedersen, T. L., Smith, S. R., Bamforth, F., Greiner, R., McManus, B., Newman, J. W., Goodfriend, T., Wishart, D. S., PLoS One 2011, 6,.

[56] Trabado, S., Al-Salameh, A., Croixmarie, V., Masson, P., Corruble, E., Fève, B., Colle, R., Ripoll, L., Walther, B., Boursier-Neyret, C., Werner, E., Becquemont, L., Chanson, P., PLoS One 2017, 12, e0173615.

[57] Bouatra, S., Aziat, F., Mandal, R., Guo, A. C., Wilson, M. R., Knox, C., Bjorndahl, T. C., Krishnamurthy, R., Saleem, F., Liu, P., Dame, Z. T., Poelzer, J., Huynh, J., Yallou, F. S., Psychogios, N., Dong, E., Bogumil, R., Roehring, C., Wishart, D. S., PLoS One 2013, 8,.

[58] Rockwell, H. E., Gao, F., Chen, E. Y., McDaniel, J., Sarangarajan, R., Narain, N. R., Kiebish, M. A., Lipids 2016, 51, 875–886.

[59] Folch, J., Lees, M., Sloane-Stanley, G., J. Biol. Chem 1957, 226, 497–509.

[60] Bligh, E. G., Dyer, W. J., Can. J. Biochem. Physiol. 1959, 37, 911–917.

[61] Retra, K., Bleijerveld, O., Van Gestel, R., Tielens, A. G., Van Hellemond, J., Brouwers, J., Rapid Commun Mass Spectrom 2008, 22, 1853–1862.

[62] Reis, a., Rudnitskaya, a., Blackburn, G. J., Fauzi, N. M., Pitt, a. R., Spickett, C. M., J. Lipid Res. 2013, 54, 1812–1824.

[63] Matyash, V., Liebisch, G., Kurzchalia, T. V, Shevchenko, A., Schwudke, D., J. Lipid Res. 2008, 49, 1137–46.

[64] Löfgren, L., Ståhlman, M., Forsberg, G.-B. G.-B., Saarinen, S., Nilsson, R., Hansson, G. I., Lofgren, L., Stahlman, M., Forsberg, G.-B. G.-B., Saarinen, S., Nilsson, R., Hansson, G. I., J. Lipid Res. 2012, 53, 1690–1700.

[65] Christinat, N., Morin-Rivron, D., Masoodi, M., J. Proteome Res. 2016, 15, 2228–2235.

[66] Sarafian, M. H., Gaudin, M., Lewis, M. R., Martin, F. P., Holmes, E., Nicholson, J. K., Dumas, M. E., Anal. Chem. 2014, 86, 5766–5774.

[67] Hamilton, J. G., Comai, K., Lipids 1988, 23, 1146–9.

[68] Kim, H., Salem, N., J. Lipid Res. 1990, 31, 2285–2289.

[69] Gross, R. W., Han, X., Chem. Biol. 2011, 18, 284–91.

[70] Madsen, R., Lundstedt, T., Trygg, J., Anal. Chim. Acta 2010, 659, 23–33.

[71] Spagou, K., Tsoukali, H., Raikos, N., Gika, H., Wilson, I. D., Theodoridis, G., J. Sep. Sci. 2010, 33, 716–727.

[72] Bennette, N. B., Eng, J. F., Dismukes, G. C., Anal. Chem. 2011, 83, 3808–3816.

[73] Lu, W., Bennett, B. D., Rabinowitz, J. D., J. Chromatogr. B Anal. Technol. Biomed. Life Sci. 2008, 871, 236–242.

[74] Chen, J., Wang, W., Lv, S., Yin, P., Zhao, X., Lu, X., Zhang, F., Xu, G., Anal. Chim. Acta 2009, 650, 3–9.

[75] KEGG PATHWAY: Metabolic pathways - Homo sapiens (human) http://www.genome.jp/kegg- bin/show_pathway?scale=1.0&query=&map=hsa01100&scale=0.35&auto_image=&show_description=hide&multi_query=&show_module_list= (accessed Nov 14, 2017